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**WORLD CONFERENCE ON PRE-ERYTHROCYTIC STAGE MALARIA VACCINE
DEVELOPMENT: CURRENT STATUS AND FUTURE PROSPECTS
APRIL 12-15, 1989, BETHESDA, MARYLAND, USA**

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

LARRY W. LAUGHLIN
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→ Contents:

ABSTRACTS/NUMERICAL LISTING

Abstract

Presenter: Clyde

1

- Protective immunity to Plasmodium falciparum and Plasmodium vivax induced by irradiated sporozoites: University of Maryland Studies, 1971-1975,

Presenter: Rieckmann

2

- Human immunization with irradiated sporozoites,

Presenter: Satterthwait

3

- The conformational restriction of synthetic peptide vaccines for malaria,

Presenter: Gordon

4

- Subunit vaccine designed to induce protective antibodies against the circumsporozoite (CS) protein of Plasmodium berghei,

Presenter: Charoenvit

5

- Subunit vaccine induced polyclonal antibodies compared with irradiated sporozoite induced monoclonal antibodies in Protein against Plasmodium yoelii,

Presenter: Zavala

6

- Induction of protective antibodies against Plasmodium berghei sporozoite by different sub-unit vaccines,

Presenter: Herrington

7

- Clinical trials of sporozoite vaccines at the University of Maryland,

Presenter: Sturchler

8

- Clinical trials with sporozoite vaccines,

Presenter: Collins

9

- Testing of Plasmodium vivax CS proteins in Saimiri monkeys,

Presenter: Hollingdale

10

- Anti-sporozoite antibodies and protection,

→ to p. 12

6. p. 117
Presenter: Mellouk 11

→ The study of exo-erythrocytic stages of malaria parasites in vitro.

Presenter: Schofield 12

→ T cell immunity to pre-erythrocytic malaria: lysis or lymphokines.

Presenter: Good 13

→ Immunogenicity of the circumsporozoite protein of Plasmodium falciparum.

Presenter: Sinigaglia 14

→ A widely recognized malaria T-cell epitope.

Presenter: Kryzch 15

→ The role of T cells in immune response against Plasmodium berghei circumsporozoite protein.

Presenter: Nardin 16

→ Cellular and humoral immune responses to a recombinant Plasmodium falciparum CS protein in sporozoite-immunized rodent hosts and human volunteers.

Presenter: Weiss 17

→ A T cell epitope recognized by CD8+ cytotoxic T cells on the Plasmodium yoelii CS protein.

Presenter: Hoffman 18

→ T cell proliferative responses to the Plasmodium falciparum CS protein in humans after immunization with a subunit vaccine and natural exposure to malaria.

Presenter: Sinden 19

→ The development of methods for routine production, and statistical evaluation of high-density cultures of the EE stages of Plasmodium berghei. (S)

Presenter: Millet	20
Use of non-human primate hepatocytes for <u>in vitro</u> study of liver stages of malaria parasites.	
Presenter: Hoffman	21
Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes.	
Presenter: Verhave	22
Studies on the fate of irradiated <u>Plasmodium berghei</u> sporozoites in three different rat strains.	
Presenter: Maheshwari	23
Role of cytokines in malaria infection.	
Presenter: Hedstrom	24
Molecular characterization of py140 the second protein identified on the surface of malaria sporozoites.	
Presenter: Drulhe	25
Identification of an additional sporozoite surface antigen <u>Plasmodium falciparum</u> also shared with liver stages.	
Presenter: Hollingdale	26
Pre-erythrocytic malaria parasites: Non-CS antigens.	
Presenter: Alkawa	27
Localization of non-CS antigens in the sporogonic stages of <u>Plasmodium yoelii</u> .	
Presenter: Marchand	28
How to select <u>Plasmodium falciparum</u> pre-erythrocytic antigens in an expression library without a Defined Probe.	

Abstract

- Presenter: Suhrbier** 29
A novel antigen specific to the liver stage of Plasmodium berghei.
- Presenter: Cochrane** 30
A Circumsporozoite-like protein is present in micronemes of mature blood stages of Malaria parasites.
- Presenter: Greenwood** 31
Immune responses to sporozoite antigens and their relationship to naturally acquired immunity to malaria.
- Presenter: Del Giudice** 32
Antibody responses to Plasmodium falciparum and Plasmodium vivax sporozoites in areas with stable and unstable malaria.
- Presenter: Webster** 33
Circumsporozoite antibody in naturally acquired Plasmodium falciparum malaria in Thailand.
- Poster: Vaughan, et al.** 34
Effect of ingested anti-sporozoite antibodies on subsequent sporozoite transmission by mosquitoes.
- Poster: Atkinson CT, Millet P, et al.** 35
Localization of circumsporozoite antigen in exoerythrocytic schizonts of Plasmodium cynomolgi.
- Poster: Bathurst, et al.** 36
Development of a recombinant vivax circumsporozoite antigen.
- Poster: Chiang, et al.** 37
Expression of malaria antigens by pseudorabies virus.

Poster: daRocha, et al.	38
<u>Plasmodium gallinaceum</u> : Inhibition of Sporozoite invasion (ISI) and EE development.	
Poster: George, et al.	39
Identification of a T cell epitope within the CS protein of <u>Plasmodium vivax</u> .	
Poster: Jaffe, et al.	40
Differences in susceptibility among mouse strains to infection with <u>Plasmodium berghei</u> sporozoites and its relationship to protection by gamma-irradiated sporozoites.	
Poster: Kumar, et al.	41
Heat shock proteins of <u>Plasmodium berghei</u> sporozoites and exoerythrocytic parasites.	
Poster: Lewis, et al.	42
Prevalence intensity and natural boosting of serum reactivity to circumsporozoite constructs of <u>Plasmodium vivax</u> , <u>Plasmodium falciparum</u> , and <u>Plasmodium brasilianum</u> .	
Poster: Lockyer	43
Variation in the <u>Plasmodium falciparum</u> circumsporozoite protein gene: Implications for vaccine development.	
Poster: Rich, et al.	44
Cell adhesion to synthetic peptides from Region II of <u>Plasmodium vivax</u> CS protein.	
Poster: Riley, et al.	45
Immune recognition of dominant T cell-stimulating epitopes from the circumsporozoite protein of <u>Plasmodium falciparum</u> and relationship to malaria morbidity in Gambian children.	
Poster: Sakhja, et al.	46
<u>Plasmodium berghei</u> exoerythrocytic merozoite surface antigen: serology and cloning.	

- Poster: Suhrbier, et al. 47
The fate of the circumsporozoite antigens and the expression of the precursor of the major merozoite antigens during the exoerythrocytic development of Plasmodium berghei.
- Poster: Sundry 48
Functional roles of T cell epitopes in the CS antigen of Plasmodium falciparum.
- Poster: Zhu J, Applah A, et al. 49
Stage-specific ribosomal RNA probes quantitate the inhibition of sporozoite invasion (ISI) assay.
- Poster: Zhu J, Aley SB, et al. 50
Plasmodium falciparum non-CS protein present in sporozoites exoerythrocytic parasites.
- Poster: Renia, et al. 51
A malaria heat shock-like protein epitope expressed on the infected hepatocyte surface is the target of ADCC mechanisms.
- Poster: van Pelt, et al. 52
Human hepatic membrane proteins 20 kD and 55 kD do specifically bind Plasmodium falciparum sporozoites.
- Poster: Mels, et al. 53
Ultrastructure of mature exoerythrocytic forms of Plasmodium falciparum in the Chimpanzee.
- Poster: Vreden, et al. 54
Boosting capacity of R16HBsAg-vaccine in mice previously infected with Plasmodium falciparum sporozoites.
- Poster: Franke ED and Lucas C 55
Humoral immune response to the circumsporozoite protein of Plasmodium vivax.

- Poster: Atkinson CT, Alkawa M, et al. 56
- Plasmodium berghei: expression of circumsporozoite protein during exoerythrocytic development in HEPG2-A16 hepatoma cells.
- Presenter: Sedegah 57
- Failure of vaccinia, pseudorabies and Salmonella CS constructs to induce protective cell-mediated immunity against sporozoites of Plasmodium yoelii.
- Presenter: Mazier 58
- Hepatic stages of malaria in culture: a helpful but limited tool to decipher protection mechanisms.
- Poster: Weiss, et al. 59
- Genetic restriction of immunity to Plasmodium yoelii sporozoites.
- Poster: Smith, et al. 60
- Designing proteosome-peptide vaccines to induce antibodies against the highly conserved pentapeptide (N1) in Region I of malaria circumsporozoite proteins: the effects of replicating the hydrophobic anchor and/or N1A.
- Poster: Lowell, et al. 61
- A recombinant Plasmodium falciparum circumsporozoite (CS) protein (R32Ft) designed with a hydrophobic decapeptide anchor: purification and immunogenicity in CS-repeat responder and non-responder mice either without adjuvants or hydrophobically complexed to proteosomes.
- Presenter: Egan 62
- Clinical trials with sporozoite vaccines - WRAIR.
- Presenter: Mellouk 63
- In vitro evaluation of a test which measures the action of antibodies on the pre-erythrocytic stages of malaria parasites.

ABSTRACTS

PROTECTIVE IMMUNITY TO PLASMODIUM FALCIPARUM AND PLASMODIUM VIVAX INDUCED BY IRRADIATED SPOROZOITES: UNIVERSITY OF MARYLAND STUDIES, 1971-1975.

David F. Clyde, University of Maryland, Baltimore, MD

The immunogenic efficacy of P. falciparum and P. vivax sporozoites was evaluated in five adult male volunteers non-immune to malaria. Inoculation of numerous sporozoites by mosquitos that had been X-irradiated within one hour previously by doses up to 17,500 rads proved safe and well tolerated. Sporozoites were rendered noninfective in 15 to 16 batch exposures when the minimum dose was 15 Krads.

Three of these volunteers during 84 days received large numbers of P. falciparum sporozoites from 379 infected mosquitos irradiated at the highest dosage, following which their immunity was challenged by homologous strain sporozoites carried by non-irradiated mosquitos. Two men developed malaria, one did not. The latter continued to be immunized with 1,062 infected irradiated mosquitos carrying homologous strain sporozoites; four additional challenges by a geographic variety of isolates of P. falciparum did not engender malaria. However, this volunteer did develop malaria when inoculated with blood stages of P. falciparum and sporozoites of P. vivax, indicating that sporozoite immunity was stage and species specific.

One volunteer was exposed to totals of 2,206 irradiated mosquitos carrying P. falciparum and 539 carrying P. vivax sporozoites. He became protected for three months and six months from the respective species. Another volunteer was exposed to 1,979 irradiated mosquitos carrying sporozoites of P. vivax, and malaria did not develop from four of five challenges.

Protection was generally reflected by rise in titer of species-specific positive circumsporozoite precipitation tests. All non-immunized volunteers acting as controls for each infective challenge developed malaria.

HUMAN IMMUNIZATION WITH IRRADIATED SPOROZOITES.

Karl H. Rieckmann, Army Malaria Research Unit, University of Sydney.

During studies conducted between 1972 and 1975, three out of ten volunteers were protected against malaria after being bitten repeatedly by mosquitoes infected with attenuated sporozoites. The immunizing sporozoites were attenuated by exposing infected *Anopheles stephensi* to X-rays at a dose of at least 120 Gy (12,000 rad). These irradiated, infected mosquitoes were allowed to feed on volunteers, thereby inoculating sporozoites into their blood stream. The three volunteers who were exposed to a total of 260 or fewer irradiated mosquitoes infected with *Plasmodium vivax* on four occasions at intervals of 2-4 weeks were not protected against subsequent challenge with the homologous strain. The four volunteers who were exposed to a total of 200 or fewer irradiated mosquitoes infected with a strain of *P. falciparum* from Ethiopia on two-four occasions over a period of one-four months were also not protected against subsequent challenge with the homologous strain.

By contrast, the three volunteers who were exposed six to eight times (over 10 to 38 weeks) to a total of 440 to 987 irradiated infected mosquitoes were protected against challenge by the homologous strain of *P. falciparum* and a heterologous strain from Vietnam. Protection lasted for at least eight weeks, but not 16 weeks, after the last immunization with irradiated sporozoites. Of particular importance is the fact that this protection was observed against parasites geographically remote from those used for immunization.

THE CONFORMATIONAL RESTRICTION OF SYNTHETIC PEPTIDE VACCINES FOR MALARIA.

Arnold Satterthwait, Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037

The effectiveness of a synthetic vaccine is dependent upon the surprising but poorly understood reaction of anti-peptide antibodies with native proteins. Peptides are largely disordered structures in water and the binding pockets of anti-peptide antibodies presumably reflect this disorder. However, in many but not all instances these antibodies bind to the surfaces of native proteins which may be ordered.

The questions are: what mechanisms lead to anti-peptide antibody-protein interactions, is the response predictable, how effective are these reactions in neutralizing infection and can we improve the result.

One approach to these questions is to compare the immune response to native and conformationally restricted peptides. To this end we are developing a new chemistry for shaping peptides and can now synthesize Type 1 reverse turns and alpha helices by replacing putative amide-amide hydrogen bonds with covalent mimics. Since every other amino acid in a protein is either in a reverse turn or helical conformation this chemistry could prove of general use.

To explore this approach we have conformationally restricted a peptide corresponding to the immunodominant epitope of the P. falciparum circumsporozoite protein. Polyclonal rabbit antiserum to the shaped malarial peptide shows a strong cross-reaction with live sporozoites demonstrating that chemically shaped peptides can serve as effective immunogens.

SUBUNIT VACCINES DESIGNED TO INDUCE PROTECTIVE ANTIBODIES AGAINST THE CIRCUMSPOROZOITE (CS) PROTEIN OF PLASMODIUM BERGHEI.

Daniel M. Gordon, James E. Egan, James D. Arthur, W. Ripley Ballou, Lawrence D. Loomis, Carl R. Alving, George H. Lowell, and Jeffrey D. Chulay. Division of Communicable Disease and Immunology, and Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington D. C. 20307-5100.

Passive transfer of monoclonal antibodies which recognize the circumsporozoite (CS) protein of Plasmodium berghei have been shown to protect C57B1/6, BALB/c, and A/J mice against subsequent challenge with viable sporozoites of the NK 65 strain or the ANKA clone of P. berghei. These data provide the basis for the hypothesis that subunit vaccines designed to induce antibodies against the CS proteins of the various human malaria sporozoites will confer protection against sporozoite-induced malaria. We have used a P. berghei rodent model to evaluate this hypothesis further. Subunit vaccines consisting of either recombinant CS proteins or various synthetic peptides have been evaluated and show varying degrees of protection against intravenous challenge with 500 to 1000 sporozoites. Methods for improving immunogenicity and efficacy using alternative delivery systems including proteosomes, liposomes, and synthetic adjuvants such as SAF have been evaluated.

SUBUNIT VACCINE INDUCED POLYCLONAL ANTIBODIES COMPARED WITH IRRADIATED SPOROZOITE INDUCED MONOCLONAL ANTIBODIES IN PROTECTION AGAINST P. YOELII SPOROZOITES.

Yupin Charoenvit, Martha Sedegah, Leo Yuan, George Lowell, Carole Cole, Rossana Bechara, Mary Leef, Mitchell Gross, Richard L. Beaudoin, and Stephen L. Hoffman, Naval Medical Research Institute, Bethesda, MD, Walter Reed Army Institute of Research, Washington, D.C., and Smith, Kilne and French Laboratories, Swedeland, PA.

The circumsporozoite (CS) protein of Plasmodium yoelii contains 19 copies of amino acids Gln-Gly-Pro-Gly-Ala-Pro (QGPGAP), eight copies of Gln-Gln-Pro-Pro (QQPP), and five copies of a degenerate nine amino acid repeat. To determine if antibodies to the repeats would protect against challenge with P. yoelii sporozoites (spz), we constructed synthetic peptide and recombinant fusion protein vaccines. Immunization with KLH-C-(QGPGAP)₄, proteosome-lauryl-C-(QGPGAP)₄ and with a fusion protein including all of the repeats (66% of the entire CS protein) induced excellent antibody responses. However, with the exception of a single experiment, these antibodies did not protect against challenge with 40-200 spz. To determine if antibodies to P. yoelii CS protein could protect against spz challenge, we passively transferred an IgG₃ Mab, NYSI (Navy yoelii sporozoite 1) into naive mice, and challenged them with spz 30 min later. A dose of 500 µg of NYSI protected 100% of mice against 5×10^3 spz, and 67% of mice against 2.5×10^4 spz. The binding of NYSI to spz is completely inhibited by incubation with (QGPGAP)₂, yet the level of antibodies to (QGPGAP)₂ and to spz by IFAT just prior to challenge was similar in the protected mice that received NYSI, and the non-protected mice immunized with the subunit vaccines. Surprisingly, the avidity of NTSI for (QGPGAP)₂ and for the recombinant protein was 5-10x lower than were the avidities of the antibodies in the sera from the non-protected mice immunized with the subunit vaccines. These data suggest that subunit vaccines based on the primary sequence of the CS protein may not produce antibodies that interact with sporozoites as effectively as do antibodies produced after immunization with the native protein which retains its conformational characteristics.

INDUCTION OF PROTECTIVE ANTIBODIES AGAINST *P. BERGHEI* SPOROZOITES BY DIFFERENT SUB-UNIT VACCINES.

F. Zavala¹, J. P. Tam², P. J. Barr³, P. J. Romero¹, V. Nussenzweig¹, and R. S. Nussenzweig¹. ¹New York University Medical Center, New York, NY, ²Rockefeller University, New York, NY, and ³Chiron Corporation, Emeryville, CA.

It has been previously demonstrated that passive transfer of monoclonal antibodies against the repeated epitopes of the CS protein of *P. berghei*, protects mice challenged with viable sporozoites. In an effort to determine whether immunization can induce the quantity and quality of circulating antibodies necessary to achieve *in vivo* protection, different subunit vaccines have been evaluated.

Immunization with a recombinant protein expressing the sequence between amino acids 81-277 of the native protein, induced antibodies in different strains of mice, though marked differences in their levels were found. Mice with a high antibody response were protected from challenge with sporozoites.

Immunization with synthetic peptides representing the repeat domain of the CS protein, coupled to tetanus toxoid, induced high levels of antibodies and abolish the infectivity of the sporozoites. Similarly high levels of protective antibodies can be achieved when immunizing with chemically defined polymers containing parasite derived T helper epitopes and without carrier proteins. The induction of protective anti-sporozoite antibodies with the polymers depends on the size of the immunogen, the molar ratio and orientation of the T and B epitopes.

CLINICAL TRIALS OF SPOROZOITE VACCINES AT THE UNIVERSITY OF MARYLAND.

Deirdre Herrington¹, David Clyde¹, Jonathan Davis¹, James Murphy¹, Genevieve Losonsky¹, David DiJohn¹, Myron Levine¹, Elizabeth Nardin², Ruth Nussenzweig², and Victor Nussenzweig². ¹ University of Maryland at Baltimore, Baltimore, MD and ² New York University, New York, NY.

The synthetic peptide P. falciparum circumsporozoite (CS) protein conjugate vaccine (NANP)₃-TT was administered to 176 volunteers in 310 doses during phase 1 and 2 studies of safety, immunogenicity and efficacy conducted over a three year period. The vaccine was safe. However, with a few notable exceptions, it stimulated only modest humoral antibody responses which were not boosted by additional immunizations. Lymphocytes from vaccinees did not proliferate when exposed in vitro to (NANP)₃. The tetanus toxoid (TT) carrier immunomodulated the response to the CS peptide in a non-predictable fashion. In North American volunteers with high pre-immunization TTs titers there was a negative correlation between TT titer prior to vaccination and subsequent anti-peptide titers, suggesting epitopic suppression. In contrast, in Venezuelan volunteers with low pre-immunization TT titers, immune enhancement was demonstrated. During efficacy challenge studies, one of seven vaccinees was protected against sporozoite challenge, although the longer prepatent period for vaccinees when compared to controls demonstrated that immune mechanisms stimulated by vaccination were capable of neutralizing a significant proportion of the sporozoite inoculum.

A phase one safety and immunogenicity study of four doses of recombinant CS protein P. vivax vaccine in 30 volunteers is in progress. The primary immunization was well tolerated in all volunteers and immunologic results are pending.

Recognition of the importance of identifying T-cell epitopes on the CS protein and including these in candidate vaccines to improve immunogenicity led us to immunize volunteers with irradiated P. falciparum sporozoites delivered by the bites of mosquitoes. The aims of these studies were to identify responder T cells, clone these cells to map the T-cell epitopes, and acquire sera and lymphocytes for use as positive controls during studies of future generations of sporozoite vaccines. Five volunteers were immunized with 54, 55, 244, 610, and 715 total infective bites delivered over a period of several months. Four of these volunteers made humoral and cellular immune responses. T cell clones are being obtained and characterized. Two volunteers who received the largest immunizing

doses were challenged by the bites of infective mosquitoes and both developed parasitemia. In the volunteer with the highest antibody titer there was a marked delay in patency as determined by serial cultures of blood.

CLINICAL TRIALS WITH SPOROZOITE VACCINES.

Dieter Sturchler, Max Just, and Michel Fernex; Hoffmann La Roche Clinical Research Unit, and University Hospital, Basle, Switzerland.

The P. falciparum sporozoite is a major target of malaria vaccine development. Recombinant and synthetic sporozoite vaccines derived from the repeat portion of the circumsporozoite (=CS) protein have been administered parenterally to humans. In challenge trials, the two vaccines offered protection to 1/6 and 1/3 volunteers, respectively. The synthetic vaccine, [NANP]₃-TT adsorbed to alum, has been given so far to 391 male and female adults and children with the objectives: to establish safety and immunogenicity, to optimize dose and adjuvant, and to determine effects of the tetanus toxoid (=TT) carrier protein on immune response to the peptide hapten. Increasing doses or concomitant administration of $0.1-1.0 \times 10^6$ U of Interferon-gamma did not enhance immunogenicity significantly, while $0.5-1.5 \times 10^6$ U of Interferon-alpha had a measurable effect on IgG anti-peptide antibody titers. Concomitantly the cellular response to TT increased significantly. Results of epitopic suppression trials are pending. P. falciparum sporozoite vaccine development has gained momentum with the reporting (Sinigaglia et al. 1988 Eur J Immunol 18:633) of a non-variant, universal T-cell epitope situated near the C-terminus of the CS protein.

TESTING OF PLASMODIUM VIVAX CS PROTEINS IN SAIMIRI MONKEYS.

William E. Collins, Malaria Branch, Centers for Disease Control, Atlanta, GA.

Recombinant proteins directed against the CS protein of Plasmodium vivax were tested for safety, immunogenicity, and efficacy in Saimiri sciureus boliviensis monkeys. Monkeys were immunized three times using either alum or muramyl tripeptide as adjuvants. Monkeys were challenged with 10,000 sporozoites of the Salvador I strain of P. vivax using sporozoites from mosquitoes infected by membrane feeding on gametocytes from chimpanzees. Antibody responses, as measured by IFA and ELISA were high. Five of 30 monkeys immunized with recombinant proteins were protected. Although their antibody responses were markedly higher, only two of six positive control monkeys immunized with irradiated sporozoites were protected. The animals appear less susceptible to sporozoite infection than would be expected in humans; however, protection following immunization was demonstrated. The predictive usefulness of this model must await the results of additional human and monkey trials with alternative adjuvants and immunizing regimens.

ANTI-SPOROZOITE ANTIBODIES AND PROTECTION.

Michael R. Hollingdale, Biomedical Research Institute, Rockville, MD.

Antibodies to malarial sporozoites are naturally acquired by individuals living in endemic areas, and can be detected by serological assays including IFA using intact sporozoites. Anti-sporozoite titers rise with age suggesting that they are protective. However, passive transfer experiments in rodents have not conclusively demonstrated their protective activity. Such antibodies predominantly react with the immunodominant repeat region of CS proteins, and MAb's to CS proteins, both intact antibodies and monovalent Fab fragments, passively protected mice to sporozoite infection even at low concentrations. The functional differences between naturally-acquired and monoclonal antibodies require investigation, as MAb-neutralizing activity may define epitopes, probably conformational-dependent, that are crucial to vaccine design. Protective anti-sporozoite antibodies may protect in the intact animal directly blocking hepatocyte recognition and invasion, by facilitating Fc-mediated macrophage (Kupffer cell) opsonization, or other mechanisms. The mechanism by which sporozoites reach hepatocytes is still poorly understood, and both direct penetration of endothelial cells, or passage through Kupffer cells, to the space of Disse have been postulated. However, once in close approximation with hepatocytes, sporozoite invasion is mediated by a complex series of steps involving CS protein non-specific (repeat region) and specific (NI region) interactions with putative hepatic 55 and 35 Kd protein receptors, CS translocation to the PVM, and processing of CS to its lowest molecular weight form. The inhibition of sporozoite invasion (ISI) in vitro assay has been shown to correlate with protective anti-sporozoite antibodies in human volunteer CS vaccine trials, although it is not designed to necessarily represent in vivo mechanisms. These points will be further discussed.

THE STUDY OF EXO-ERYTHROCYTIC STAGES OF MALARIA PARASITES IN VITRO.

Richard L. Beaudoin, Sylvie Mellouk and Gary Long. Naval Medical Research Institute, Bethesda, MD, USA.

Attempts at growing the exo-erythrocytic parasites in culture go back almost as far as the realization that such a cycle existed. Since the discovery of the EE cycle of avian malaria predates the demonstration of these parasites in mammalian hosts by at least a decade, it is not surprising that a continuous culture system was first developed for the study of the avian parasite, Plasmodium fallax. This system for culturing EE parasites was dependent on the establishment of a continuous embryo passage in which tissue containing infected cells were grafted onto the chorioallantoic membrane of a new generation of embryos. These infected embryos were then the source of primary cultures. The approach obviated one of the main problems in culturing these parasites, that of maintaining sterility of the infective inoculum. In first attempts at establishing cultures of the mammalian parasites we tried to emulate the success we had achieved with the bird plasmodia by adopting a similar approach. After three frustrating years without success, we discovered that the mosquito itself could be rendered aseptic by thoroughly washing its surface with a disinfectant thereby providing a sterile sporozoite inoculum. This discovery coupled with advances in the cultivation of functional hepatocytes then led to a new approach which developed into the system in use today. These early successes in growing P. berghei in a variety of cells, have been multiplied and expanded upon by many of the participants here today until the system can now be used to grow all four human parasites. Because it is now clear that the EE parasites are a major target of the immune system, we are presently adapting the cultures to meet the immunological objectives of vaccine development. These initiatives include the design of target cells for measuring cytotoxic T cell activity and validation of tests like HILDA. In assessing the former, maturation in mouse cells is necessary for studies with the rodent model since assays of CTL activity must be performed in the context of major histocompatibility antigens. In this regard, we have developed two mouse hepatocyte cell lines, one from a primary hepatocyte culture, the other from the fusion of a mouse hepatocyte with a continuous rat liver line. Both of these lines support maturation of liver parasites with release of EE merozoites. The HILDA assay measures the efficiency of individual components of the immune response and when properly interpreted correlates reasonably well with the expected contribution to a protective response of the components being evaluated.

T CELL IMMUNITY TO PRE-ERYTHROCYTIC MALARIA: LYSIS OR LYMPHOKINES?

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In the Plasmodium berghei rodent malaria model, the sterile immunity induced by attenuated sporozoites depends upon the antigen-specific sensitization of CD8⁺ T cells. Such T cells may exert their effect via cytotoxic lysis of infected target cells (hepatocytes), by recognition of malarial antigens in association with MHC Class I; or via other routes of activation which result in the elaboration of parasiticidal lymphokines. Strong evidence for the latter postulate comes from our observation that neutralization of endogenous IFN-gamma in both rats and mice reverses acquired cellular immunity to sporozoites. The implications of these findings will be discussed in the light of current models of CD8⁺ T cell activity.

IMMUNOGENICITY OF THE CIRCUMSPOROZOITE PROTEIN OF PLASMODIUM FALCIPARUM.

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The circumsporozoite (CS) protein covers the sporozoite surface and is a vaccine candidate for malaria since antibodies against the protein as well as T cells (probably cytotoxic T cells) specific for the protein are able to protect against malaria in model systems. We have studied the immunogenicity of the protein in humans and H-2 congenic mice. Mice were immunized with a CS (7G8) recombinant vaccinia virus or with purified CS protein (7G8 clone) derived from recombinant baculovirus infected cells. Nine different mouse strains were immunized with the vaccinia construct and five different strains were immunized with the purified CS protein. The results of both experiments were directly comparable and revealed that most mouse strains are either low responders or, in some cases, non-responders. Mice bearing the I-A^b or I-A^k alleles of the MHC were the highest responders. The results of experiments in FI mice revealed that responsiveness is dominant, suggesting that 'suppressor genes' are not responsible for the poor immunogenicity in these experiments. Similarly, we have observed that the sera from many humans naturally and chronically exposed to sporozoites do not contain CS-specific antibodies but that those negative sera do contain antibodies to crude malaria antigen and often contain antibodies specific to sexual or asexual stage protein. These data are consistent with a major role for human *I_r* genes in the response to this protein. Definition of T cell epitopes one recognized by both humans and mice reveals that the polymorphic regions of one the protein are immunodominant. These observations highlight the importance of T cells in sporozoite immunity and should be taken into account in vaccine design.

A WIDELY RECOGNIZED MALARIA T-CELL EPITOPE.

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An ideal vaccine should elicit a long lasting immune response against the natural parasite, both at the T- and B-cell level. The immune response should occur in all individuals and be directed against determinants that do not vary in the natural parasite population. During the characterization of epitopes of the malaria parasite Plasmodium falciparum that are recognized by human T cells, we analyzed a sequence of the circumsporozoite protein (CS protein residues 378-398 with Cys replaced by Ala), and found that synthetic peptides corresponding to this sequence are recognized by T cells in association with many different MHC class II molecules, both in mouse and in man (Sinigaglia et al. *Nature*, **336**: 778, 1988). This region of the circumsporozoite protein is invariant in different parasite isolates. Mice preimmunized with the CS 378-398 peptide had increased anti-parasite responses following immunization with sporozoites, suggesting that inclusion of this sequence in a vaccine would allow boosting of antibody responses following natural infection. A vaccine consisting of the CS 378-398 peptide linked to an appropriate B-cell epitope might well induce both humoral and cellular parasite-specific immunity in the genetically diverse human population.

**THE ROLE OF T CELLS IN IMMUNE RESPONSE AGAINST P. BERGHEI
CIRCUMSPOROZOITE PROTEIN.**

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The development of efficacious vaccines against malaria requires an understanding of the mechanisms involved in protective immunity. Previous studies with Plasmodium demonstrated that sporozoite immunity is dependent upon both antibody responses specific for the repeat region of the circumsporozoite (CS) protein and T cell responses specific for non-repeat regions. In this study we analyzed murine splenic and lymph node T cell repertoires specific for epitopes on the P. berghei CS protein. T cells, derived from H-2^b H-2^d, and H-2^k haplotypes, primed with sporozoites, recombinant CS protein or synthetic CS peptides were tested for antigen specificity in proliferative T cell assay and for cytolytic activity. The CS protein-specific T cell repertoire differed according to the haplotype of each murine strain tested: H-2^d mice were high responders to sporozoites in culture, whereas H-2^b and H-2^k were low responders. Proliferative responses varied also according to the haplotype's protective status against malaria infection. The form of the priming antigen appeared to influence the pathway of antigen processing and presentation to T cells; intact sporozoites required activated B cells as antigen presenting cells and generally did not prime for CS peptide activity. Priming with recombinant CS protein, on the contrary generated T cells responsive to CS protein peptides in vitro. Parallel analysis of T cell repertoire expressing cytolytic T cells activity is in progress. These studies provide new insight into the role of CS protein-specific T cell repertoires in protective immunity to malaria.

CELLULAR AND HUMORAL IMMUNE RESPONSES TO A RECOMBINANT P. falciparum CS PROTEIN IN SPOROZOITE-IMMUNIZED RODENT HOSTS AND HUMAN VOLUNTEERS.

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The immune response of sporozoite-immunized rodents and of human volunteers immunized by multiple exposures to the bites of irradiated P. falciparum infected mosquitoes has been investigated using yeast-derived recombinant P. falciparum CS (rPfCS) protein. The murine immune response to immunization with rPfCS was not genetically restricted. Nine different murine haplotypes, when immunized with rPfCS, developed high levels of ant sporozoite antibodies detectable by IFA and RIA. Injection of rPfCS induced a secondary antibody response in sporozoite-pruned mice.

Immune cells obtained from P. falciparum sporozoite-immunized rodent or human hosts proliferated when challenged *in vitro* with rPfCS. Murine T cell epitopes were mapped in the C terminal region of the CS protein using overlapping synthetic peptides. The fine specificity of the human T cell response was investigated using T cell clones derived from PBL of a sporozoite-immunized volunteer. A total of 40 CD4+ T cell clones were obtained. Stimulation indices ranged from 2.5 to 103.4 following challenge with rPfCS in the presence, but not the absence, of antigen presenting cells. The clones were specific for rPfCS and did not proliferate when challenged with yeast-derived recombinant P. vivax or P. berghei CS protein or with a yeast extract control. The clones also recognized the native CS protein in extracts of P. falciparum, but not P. berghei or P. cynomolgi, sporozoites. IL-2 and gamma interferon were produced by the clones following antigen stimulation *in vitro*.

A T CELL EPITOPE RECOGNIZED BY CD8+ CYTOTOXIC T CELLS ON THE P. YOELII CS PROTEIN.

Walter R. Weiss, Richard Houghten, Jay Berzofsky, Michael Hollingdale, Louis H. Miller, Michael F. Good, and Stephen L. Hoffman. Naval Medical Research Institute, Bethesda, MD, Scripps Clinic, La Jolla, CA, National Institutes of Health, Bethesda, MD, and Biomedical Research Institute, Rockville, MD.

When BALB/c mice are immunized with P. yoelii sporozoites, the major immune effector mechanism is the CD8+ T cell. Using short synthetic peptides, we have found a T cell epitope on the P. yoelii CS protein which is recognized by CD8+ CTL from immune BALB/c mice. We will describe our screening technique and discuss our attempts to relate this epitope to the protective T cell response.

T CELL PROLIFERATIVE RESPONSES TO THE P. FALCIPARUM CS PROTEIN IN HUMANS AFTER IMMUNIZATION WITH A SUBUNIT VACCINE AND NATURAL EXPOSURE TO MALARIA.

Stephen L. Hoffman, Charles N. Oster, Carl Mason, James Sherwood, W. Ripley Ballou, Mutuma Muganbi, Nancy Hentzel, Robert J. Hartzman, and Jeffrey D. Chulay. Naval Medical Research Institute, Bethesda, MD, Walter Reed Army Institute of Research, Washington, DC, and U.S. Army Medical Research Unit and Kenya Medical Research Institute, Nairobi, Kenya.

We have conducted a series of lymphocyte proliferation assays to better understand the T cell response to subunit CS protein vaccines and naturally acquired malaria. Lymphocytes from 11 of 13 human volunteers immunized with three doses of R32tet32 at one month intervals proliferated after exposure to R32tet32, but only 3 of 13 to a recombinant CS protein including only two non-CS protein amino acid residues (R32LR), suggesting a highly restricted response to the CS protein repeat region. Six of these volunteers, none of whom had previously responded to R32LR were boosted with R32tet32 12 months later, and lymphocytes from three of the six (the three with the highest levels of antibodies to CS protein) responded to R32LR, indicating that the restriction was not as severe as first suggested. After demonstrating that there were adequate T helper cell sites on the P. berghei CS protein to completely overcome genetic restriction to the repeat region, we looked for analogous T helper sites on the P. falciparum CS protein. Studies in humans in Kenya revealed a limited response to R32LR, but at least five non-repeat region T cell proliferative sites on the P. falciparum CS protein. The proliferative response to two of these sites, which included residues 361-390, correlated with resistance to infection with P. falciparum. HLA analysis of responders and non-responders to residues 361-380, and of protected and non-protected individuals suggests a correlation with several Class I HLA alleles.

**THE DEVELOPMENT OF METHODS FOR ROUTINE PRODUCTION, AND
STATISTICAL EVALUATION OF HIGH-DENSITY CULTURES OF THE EE STAGES OF
PLASMODIUM BERGHEI.**

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Following the successful introduction of Plasmodium berghei EE stages *in vitro* (Strome et al, 1979) significant improvements were achieved by the use of a variety of primary hepatocytes and host cell, including the cell line HepG2 A16 (Hollingdale et al, 1983). Despite these improvements we considered these cultures to be less than ideal for a variety of reasons including: 1. The rate of growth and size of the EE parasite was less than that observed *in vivo*; 2. Parasite density was too low and variable to allow either biochemical/immunological study, or statistically robust analysis of immunological and chemotherapeutic intervention strategies; 3. Methods of analysis of parasite growth were inadequate.

We will report results of an ongoing analysis of methods for the improvement of P. berghei EE culture in which we have examined a variety of new host cell types, media supplements, parasite parameters, and technical aspects of cell culture. Currently cultures are routinely achieving 20% of host cells infected (this can be raised to 50% under specific conditions), in quantities that allow direct induction of specific anti-liverstage immune response (see Suhrbier et al, this meeting) and which permit biochemical and cell biological studies. The scale and reproducibility of parasite growth permits stringent numerical analysis of both drug and immune inhibitors of parasite growth. A method for a rapid and accurate assessment of parasite growth in large numbers of replicate cultures, using a subtelomeric DNA probe will be further illustrated by examples from chemotherapeutic experiments.

The integration of the EE culture system with blood stage cultures to include significant invasion of rodent erythrocytes within which the entire asexual and sexual development is obtained will be described. Thus the entire vertebrate phase of this mammalian malarial parasite is now possible *in continuo*. The extension of these methods to other species could have significant application.

Acknowledgement: We gratefully acknowledge the financial support of the Medical Research Council of Great Britain, and the cooperation of Prof. C. Frontali for recombinant DNA probes.

USE OF NON-HUMAN PRIMATE HEPATOCYTES FOR IN VITRO STUDY OF LIVER STAGES OF MALARIA PARASITES.

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Primary cultures of simian hepatocytes have been recently used to study liver stages of primate malaria parasites. Methods were developed that allow invasion of Plasmodium cynomolgi sporozoites and maturation to schizont stages in rhesus monkey hepatocytes. This system was used to demonstrate strain specific inhibition of schizont development with monoclonal antibodies (Mabs) produced against the CS protein. Species cross-reactivities were also shown in this inhibition essay. Immunoelectron microscopy, using gold-labelled Mabs and cultured parasites demonstrated that CS protein persists in seven-day old liver stages. A rhesus monkey was immunized with autologous hepatocytes (collected by biopsy) infected in vitro with liver stages of P. cynomolgi. This immunization elicited antibodies reacting with sporozoite, liver stage, and blood stage parasites. In addition to P. cynomolgi, the in vitro system has been adapted to various simian (P. knowlesi, P. inui, P. coatnei) and human (P. malariae and P. vivax) parasites. In particular, liver stages of P. vivax have been reproducibly cultured in hepatocytes from Saimiri monkeys. This model was used to study activity of sera from immunization trials conducted with Saimiri monkeys immunized with recombinant P. vivax CS proteins. Pre- and post-immunization sera were tested for their ability to inhibit P. vivax liver stage development in vitro. Post-immunization sera inhibited the parasite development (90 to 100% inhibition compared to pre-immunization and control monkey sera), thus demonstrating the induction of antibodies effective against sporozoites. No relationship, however, was detected between in vitro inhibition and in vivo protection or antibody titers detected by IFA or ELISA. Supported by USAID PASA BST-0453-P-HC-2086-03 and NRC Fellowship to PM.

**SPOROZOITE VACCINE INDUCES GENETICALLY RESTRICTED T CELL
ELIMINATION OF MALARIA FROM HEPATOCYTES.**

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The target of the T cell-dependent immunity that protects mice immunized with irradiation attenuated malaria sporozoites has not been established. We report that immune BALB/c mice develop malaria specific, and CD8+ T cell-dependent inflammatory infiltrates in their livers after challenge with Plasmodium berghei sporozoites, and that spleen cells from immune BALB/c and C57BL/6 mice eliminate hepatocytes infected with the liver stage of P. berghei from in vitro culture. The activity against infected hepatocytes is not inhibited by anti-interferon γ and not duplicated by culture supernatants. It is genetically restricted, indicating that malaria antigens on the hepatocyte surface are recognized by immune T effector cells. Further subunit vaccine development will require identification of the antigens recognized by these T cells, and a method of immunization that induces such immunity.

**STUDIES ON THE FATE OF IRRADIATED PLASMODIUM BERGHEI SPOROZOITES
IN THREE DIFFERENT RAT STRAINS.**

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It has been shown more than 20 years ago that irradiated sporozoites induce protective immunity to sporozoite challenge in mice. The mechanism by which irradiated sporozoites induce immunity and the fate of irradiated life sporozoites is only fragmentarily known. The interaction of irradiated sporozoites with host cells has been studied *in vitro* suggesting that they were able to transform but did not start nuclear division, which commences normally at 20–22 h. We have extended these studies to the *in vitro* situation comparing γ -irradiated sporozoite (8kRad) infectivity and development with normal infective sporozoites in three different rat strains. At 46 h post inoculation, the livers were removed and fixed for electron microscopy and light microscopy. It has been shown previously, Brown Norway rats were the most susceptible (235 parasites/cm²). Compared to Wistar and Sprague-Dawley rats (55/cm²). Only blood drawn from the control Brown Norway rat at autopsy was infectious to mice. In the livers of rats receiving irradiated sporozoites, no mature liver stages could be detected with routine light ant sporozoite antiserum, in IFA small fluorescing stages were seen at 46 h suggesting the persistence of undeveloped sporozoites. Electron microscopy should confirm the parasitic nature of these bodies. The roles of these remaining parasites in immunity to sporozoite infection will be discussed.

ROLE OF CYTOKINES IN MALARIA INFECTION

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We have tested the prophylactic effect of Escherichia coli derived recombinant human gamma interferon (rHuIFN- γ) against sporozoite- or trophozoite-induced Plasmodium cynomolgi B malaria infection in rhesus monkeys. The course of the infection of rhesus monkeys with P. cynomolgi B closely resembles that of relapsing P. vivax malaria in humans. Data show that treatment with only three doses of rHuIFN- γ (0.1 mg/kg body weight) given on days -1, 0, and +1 after infection protected monkeys against sporozoite-induced P. cynomolgi infection. Animals initially protected by rHuIFN- γ treatment remained susceptible to reinfection. No inhibitory effect of rHuIFN- γ was seen against trophozoite-induced infection. We have also tested the effect of recombinant human tumor necrosis factor (rHuTNF) in rhesus monkeys. No significant activity of TNF was seen against trophozoite-induced P. cynomolgi B infection. We have also shown that rHuIFN- γ inhibited schizogony in functional human hepatocytes infected with P. falciparum sporozoites. These results suggest that the inhibitory effect of IFN is limited to the exoerythrocytic stage of parasite development. Interleukin-1 (IL-1) also inhibited hepatic development of P. falciparum sporozoites; however, IL-1 treatment was effective only when applied before sporozoite inoculation.

MOLECULAR CHARACTERIZATION OF py^{140} THE SECOND PROTEIN IDENTIFIED ON THE SURFACE OF MALARIA SPOROZOITES.

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Our recent studies indicate that the circumsporozoite (CS) protein is not the only antigen on the surface of Plasmodium yoelli sporozoites. Investigations to distinguish parasite molecules involved with the protective property of the irradiated sporozoite vaccine produced a monoclonal antibody [Navy yoelli sporozoite 4 (NYS4)], that binds not to the CS protein of P. yoelli but to a distinct sporozoite protein of 140 kD ($py140$). Like NYSI, a CS protein specific monoclonal antibody, NYS4 adheres to the surface of motile sporozoites and to the material which is shed by the live parasites in vitro. Intrigued by the possibility that the sporozoite surface is composed of more than a single protein, we isolated a genomic P. yoelli λ gt10 clone (M4) expressing the NYS4 antigen. Clone M4 contains a 2.2kB DNA insert which does not hybridize to the isolated CS protein gene but does hybridize at high stringency to the DNAs from three P. yoelli parasite clones. On the other hand, the inferred amino acid sequence of the M4 peptide displays features that are similar to the CS protein: at least two repeating amino acid sequences (3-mer and 6-mer) and a Region II-like domain. The NYS4 antibody recognizes a synthetic 18 amino acid peptide which consists of 3 of the 6-mer repeats. These findings demonstrate that at least two different proteins form the surface of P. yoelli sporozoites and that both are accessible candidates for vaccine development.

IDENTIFICATION OF AN ADDITIONAL SPOROZOITE SURFACE ANTIGEN OF P. FALCIPARUM ALSO SHARED WITH LIVER STAGES.

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The circumsporozoite protein of Plasmodium falciparum has been considered up to now to be the only antigen expressed on the surface of sporozoites. However, on the basis of evidence from: 1) results of competition assays between sera from endemic areas and both Mabs and CS repeat peptides; 2) the ability of these same sera to recognize the CS in sporozoite extracts; and 3) the pattern of reactivity of human Mabs with sporozoites from different isolates, it was concluded that there are other sporozoite surface antigens recognized by the immune system. To better characterize these, a subset of 120 previously isolated DNA clones thought to correspond mainly to pre-erythrocytic antigens was screened further. For this purpose, we used polyclonal but highly selected antibodies, that is human sera containing antibodies reactive with the surface of sporozoites by IFA but unreactive with the CS antigen. Using this approach, we have now identified a DNA clone encoding an 87 aa non-repetitive polypeptide. Its structure has no homology with known P. falciparum proteins including CS and the corresponding gene is located on chromosome-2. Antibodies affinity-purified on the recombinant product recognize a 70kDa protein detected in sporozoites of some but not all of the isolates tested. In addition, when expressed, the antigen is present on the surface of only some sporozoites (e.g. some sporozoites of the NF54 strain but not sporozoites of 3D7 a clone of that strain). The location of this antigen on the sporozoite surface was first shown by the wet IFA assay and confirmed by immuno-gold labeling of ultrathin sections of sporozoites. The antigen is also expressed in P. falciparum liver stages but not detected in sporozoites of other species and was not recognized by sera from heterologous infections. A high prevalence of antibodies to this Sporozoite and Liver Stage Antigen (SALSA) was found in subjects of various age groups even in areas of very low endemicity.

PRE-ERYTHROCYTIC MALARIA PARASITES: NON-CS ANTIGENS.

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Immunization with live or irradiated sporozoites, either naturally in humans or experimentally in animals, elicits a strong antibody response that primarily recognizes circumsporozoites (CS) proteins, and that is mainly directed to the CS repeat region. The immunodominance of CS proteins mediated by the repeat region has made investigation of other relevant sporozoite antigens difficult. The paucity of exoerythrocytic (EE) material available for immunization has similarly hindered efforts to characterize EE antigens. CS proteins have been detected in EE parasites, and both polyclonal and MAb's to many blood stage antigens also react with EE parasites. However, two fundamental questions remain to be answered namely, are there other non-CS sporozoite antigens and are there non-CSEE antigens, relevant to the induction of protective immunity. Both these questions are currently being investigated. The Plasmodium falciparum liver stage specific antigen (LSA, Guerin-Marchandet al 1987) has been shown to be also associated with sporozoites and to elicit antibodies reactive in the ISI assay. Previously described cross-protection studies in mice, in which P. falciparum sporozoites, but not P. falciparum CS proteins, elicit protection to challenge with P. berghei sporozoites, suggest that a non-CS protein protective antigen common to both species is associated with sporozoites, and this is being further characterized. Other antigens under study include heat shock proteins, and C2GI, and EE merozoite major surface antigen. This project is funded by USAID contract DPE-0453-C-00-3051-00.

**LOCALIZATION OF NON-CS ANTIGENS IN THE SPOROGENIC STAGES OF
PLASMODIUM YOELII.**

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Charoenvit and associates described monoclonal antibodies directed against sporozoites of P. yoelii that recognize two non-CS proteins. The antigen recognized by NYS2 is a 140 kDa protein with a common determinant with the CS antigen. The 140 kDa antigen, also recognized by NYS4, did not become abundant until late in sporogony. This antigen was associated with the surface of budding sporozoites, but was found most abundant with endoplasmic reticulum and micronemes. A second non-CS antigen identified by NYS5 first appeared in five to seven day old oocysts, although labeling was sparse. Once sporozoite formation began, a few gold particles were seen on the plasma membrane of cytoplasmic clefts and budding sporozoites as well as associated micronemes. Immuno-electron microscopy demonstrated, therefore, that non-CS surface antigens of P. yoelii oocysts and sporozoites do occur but that they are less abundant than CS protein. Since we do not yet know the role played by non-CS antigens in the biology of the parasite, further characterization of their function is needed before their potential as vaccine candidates can be determined.

**HOW TO SELECT *PLASMODIUM FALCIPARUM* PRE-ERYTHROCYTIC ANTIGENS
IN AN EXPRESSION LIBRARY WITHOUT A DEFINED PROBE.**

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The restricted access to *P. falciparum* liver stages have greatly limited the analysis of the antigenic content of that stage. Due to the lack of material to perform immunochemical studies, of access to mRNA, and of monoclonal probes, we decided to screen a genomic library with stage-restricted human antibodies. This strategy led to the identification of a large number of DNA fragments encoding both sporozoite specific as well as liver-stage specific epitopes. Following the initial characterization of one liver stage antigen, further screening was performed by using additional selective human antibodies. These were defined as having a high degree of reactivity with native antigens on either of the two stages while being negative with the already known molecules of these same two stages. From this second screening and the study of cross-reactions, several subsets of DNA clones expressing antigens present on the surface of sporozoites, or in liver stages, or in both, could be identified. In exposed individuals, a high prevalence of antibodies to several of these antigens was found.

A NOVEL ANTIGEN SPECIFIC TO THE LIVER STAGE OF PLASMODIUM BERGHEI.

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A variety of immunization protocols, using live sporozoite inocula followed by drug treatment and/or cultured liver schizonts as antigen, was used to establish a panel of monoclonal antibodies reacting with 1) host liver cells, 2) sporozoites and liver stages, 3) blood and liver stages, and 4) liver stages alone. Data will be presented on one of the monoclonal antibodies specific for liver stages of P. berghei for which the target antigen has been partially characterized and with which passive protection experiments have been initiated.

The target antigen of this monoclonal antibody is not present on the sporozoite but first appears one-two hours after invasion of the hepatocyte. During the trophozoite and schizont stages it appears to localize to the parasitophorous vacuole membrane (PVM) and in the free mature segmenter (48-60 hours) had a freckled distribution consistent with the localization of the disrupted PVM. The target antigen was not detected on liver stage merozoites or blood stage parasites.

A CIRCUMSPOROZOITE-LIKE PROTEIN IS PRESENT IN MICRONEMES OF MATURE BLOOD STAGES OF MALARIA PARASITES.

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We demonstrate for the first time the presence of a circumsporozoite (CS)-like protein in invasive blood stages of malaria parasites. Immunogold electron microscopy using antisporeozoite monoclonal antibodies localized these antigens in the micronemes of merozoites. Western immunoblot and two-dimensional gel electrophoresis of mature blood stage extracts of Plasmodium falciparum, P. berghei, P. cynomolgi, and P. brasiliense identified polypeptides having the same apparent molecular mass and isoelectric points as the corresponding sporozoite (CS) proteins. The CS-like protein of merozoites is present in relatively minor amounts compared to the CS protein of sporozoites. Mice with long-term P. berghei blood-induced infections develop antibodies which react with sporozoites.

IMMUNE RESPONSES TO SPOROZOITE ANTIGENS AND THEIR RELATIONSHIP TO NATURALLY ACQUIRED IMMUNITY TO MALARIA.

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Methods by which the relationship between the immune response to a malaria antigen and the development of naturally acquired immunity to malaria can be studied will be reviewed. These include comparisons between infected and non-infected subjects, study of the age profile of individual immune responses and longitudinal cohort studies. The latter provide the most useful data but they are, nevertheless, subject to many confounding factors.

Several studies of the influence of antibodies to CSP on susceptibility to malaria have been done. These provide little evidence to suggest that these antibodies, at a level achieved as a result of natural infections, are protective. Similar studies of the relationship between cell-mediated immune responses to CSP peptides and protective immunity are in progress.

Field studies are unlikely to be able to show convincingly that a specific response is an important component of naturally acquired protective immunity; they are more likely to be able to demonstrate that a particular response is unlikely to be associated with protection.

ANTIBODY RESPONSES TO PLASMODIUM FALCIPARUM AND PLASMODIUM VIVAX SPOROZOITES IN AREAS WITH STABLE AND UNSTABLE MALARIA.

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Availability of synthetic and recombinant peptides reproducing the repetitive regions of circumsporozoite (CS) proteins of P. falciparum and P. vivax has allowed both the development of assays for the detection of specific antibodies, and of potential subunit vaccines. Knowledge of the immune responses to malaria sporozoites represents a prerequisite for the optimal design of a sporozoite antigen-based vaccine. Studies carried out in areas with stable P. falciparum malaria (Tanzania) have shown that antibodies against the synthetic peptide (NANP)₄₀ increase as a function of age. Cluster analysis revealed marked inter-household variation of the anti-sporozoite antibody response, despite comparable risks of exposure to infectious bites. Similarly, an age-related prevalence of anti-sporozoite antibodies has been observed in an area of Sri Lanka with unstable malaria, using a 45-amino-acid synthetic peptide reproducing a defined sequential array of the two main 9-mer variants of the P. vivax CS protein. In this area anti-(NANP)₄₀ antibodies became detectable after the first epidemic of P. falciparum malaria. Interestingly, their prevalence also increased with age. Since this population never experienced P. falciparum malaria before, one can suggest that anti-sporozoite antibodies reflect the relative exposure to infectious bites in the different age groups, and in turn of the transmission of the disease. This is particularly useful in areas where entomological indices of transmission tend to be unreliable because of the low vectorial capacity and wide fluctuations in vector densities.

CIRCUMSPOROZOITE ANTIBODY IN NATURALLY ACQUIRED PLASMODIUM FALCIPARUM MALARIA IN THAILAND.

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Antibody (Ab) to sporozoites of P. falciparum (PF) is made against an immunodominant B cell epitope in the repeat region of the circumsporozoite (CS) protein. We have studied the antibody response to sporozoites in naturally acquired PF malaria in occupational and endemic populations. Thai soldiers experiencing symptomatic malaria were found to CS Ab responses of variable low magnitude which peaked following detection of parasitemia and had a serum half-life of less than one month. Boosting of the antibody response was infrequent. Classical Ab class switching from IgM to IgG was not observed. In oligosymptomatic endemic villagers CS Ab prevalence and concentration paralleled seasonal variation in transmission. CS Ab levels increased with age and slowly developed over decades of life. In about 20% of infected individuals there was no detectable CS Ab response. HLA-DR typing showed no evidence for class II MHC restriction as a factor in hyporesponsiveness to CS antigen. Analysis of endemic villagers showed that adults who were persistent CS Ab responders and non-persistent responders were at comparable risk of developing parasitemia. These observations raise questions about the role of CS Ab in natural infection and about whether CS Ab even if stimulated to higher levels by immunization would be protective. Endemic villagers tended to have low density parasitemias year-round despite seasonal peaks in mosquito transmission suggesting an effective limiting immunity-directed mostly against the asexual blood stage. Even so CS Ab may have a role in lowering the number of infective sporozoites thereby reducing the chance of superinfection.

**EFFECT OF INGESTED ANTI-SPOROZOITE ANTIBODIES ON SUBSEQUENT
SPOROZOITE TRANSMISSION BY MOSQUITOES.**

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When ingested by malaria-infected mosquitoes, anti-sporozoite antibodies (IgG) cross the mosquito midgut and interact with malaria parasites developing within the mosquito hemocoel. To determine the effect that ingested anti-sporozoite antibodies have upon subsequent sporozoite transmission, we maintained P. berghei-infected A. stephensi mosquitoes on anti-sporozoite antibodies (i.e., immune mice) throughout parasite sporogony. Control mosquitoes were also infected but were maintained on naive (i.e., nonimmune) mice. When transmitted via infectious mosquito bite, sporozoites from immune-fed mosquitoes were able to infect naive mice more efficiently than were sporozoites from nonimmune-fed control mosquitoes, as determined by the time elapsed before detection of blood parasitemias (patency). Furthermore, sporozoites transmitted via bite by immune-fed mosquitoes successfully infected sporozoite-immune mice (ELISA titers > 1:3200) whereas sporozoites transmitted by control mosquitoes did not. Thus, ingestion of anti-sporozoite antibodies by infected mosquitoes seemed somehow to produce more virulent mosquito-borne sporozoites. These data demonstrate the importance of incorporating vector studies as an integral part of vaccine development to detect possible side effects that vaccine-induced antibodies may have upon parasite sporogony in the mosquito.

LOCALIZATION OF CIRCUMSPOROZOITE ANTIGEN IN EXOERYTHROCYTIC SCHIZONTS OF PLASMODIUM CYNOMOLGI.

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Colloidal gold probes and post-embedding immunoelectron microscopy were used to localize circumsporozoite (CS) antigen in cultures of P. cynomolgi exoerythrocytic (EE) schizonts. Both small uninucleate and large were found in five-day-old cultures. A mouse monoclonal antibody to the repeat region of the P. cynomolgi CS protein labeled the plasma membrane and surface of small and large EE schizonts as well as the surrounding parasitophorous vacuole membrane and space. Density of labeling decreased significantly as EE schizonts increased in size and maturity. Mature eight-day-old EE schizonts with developing merozoites had little detectable labeling. Label was not observed in the host cell cytoplasm or on the surface of infected hepatocytes, indicating that epitopes associated with the repeat region of the P. cynomolgi circumsporozoite protein are sequestered within infected host cells during EE development. Standard transmission electron microscopy demonstrated that distinct populations of small, undividing EE parasites and large multinucleated EE schizonts were present in 5- and 9-day-old cultures. The small, uninucleated EE parasites meet morphological criteria described for malarial hypnozoites.

DEVELOPMENT OF A RECOMBINANT VIVAX CIRCUMSPOROZOITE ANTIGEN.

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Three different Plasmodium vivax circumsporozoite antigens have been expressed at high levels in yeast. These recombinant antigens have been purified and used in immunogenicity studies in mice, guinea pigs, and rabbits. Also, using an alum adjuvant, two of the three molecules gave significant protection in a Saimiri monkey challenge system. Antibodies from the same two antigens also performed well in both the fixed sporozoite recognition assay and the sporozoite liver invasion assay.

EXPRESSION OF MALARIA ANTIGENS BY PSEUDORABIES VIRUS.

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The use of liver recombinant Pseudorabies Virus (PRV) as a malaria vaccine to protect mice against challenge with Plasmodium berghei and Plasmodium yoelli was investigated. We have constructed a number of PRV recombinants containing the circumsporozoite CS genes from P. berghei and P. yoelli under the control of the PRV glycoprotein (gp) X promotor. The foreign genes were inserted into PRV which have been attenuated for mice. The CS genes were expressed as coding region fusions to various portions of the PRV gpX and gpIII genes. We have determined the level of expression for these recombinants in vitro as well as their ability to sero-convert mice to the CS antigen. The various constructs display varying degrees of in vitro expression which correlate well with their ability to sero-convert mice.

PLASMODIUM GALLINACEUM: INHIBITION OF SPOROZOITE INVASION (ISI) AND EE DEVELOPMENT.

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Mammalian Plasmodium sporozoites are covered with CS proteins which are detected in western blots with MAb's as higher molecular weight precursors and putative lower molecular weight surface processed form. MAb's to CS repeat regions inhibit sporozoite invasion (ISI) of cultured cells. CS protein is also found in exoerythrocytic (EE) parasites and may also occur on the surface of EE merozoites, although mice immunized with sporozoites are susceptible to infection with EE merozoites. Whereas mammalian malarias undergo only one EE cycle of development, avian malarias develop EE merozoites that can reinvade cultured cells, giving repeated cycles of EE parasites. Recently, (Krettli et al 1988) CS proteins of P. gallinaceum sporozoites have been identified, and MAb's to these CS proteins inhibited P. gallinaceum invasion of cultured chick macrophages. In vitro cultures of P. gallinaceum EE parasites have been established in a chick embryo cell line in order to further study the immunological significance of CS proteins on avian sporozoites and EE parasites and merozoites. P. gallinaceum sporozoites invaded the chick line cultured in minimal essential medium, and developed into EE trophozoites at 24 hr, EE schizonts containing merozoites at 48 hr, and a second type of larger EE parasite that developed by 72 hr. CS proteins were detected using MAb's on sporozoites, EE parasites and merozoites, although the larger 72 hr EE parasites were weakly stained. Further studies will use this system to quantitate the ISI assay. MAb's to P. gallinaceum CS also fluoresced with P. berghei sporozoites, and by western blot recognized the lower molecular weight form of P. berghei CS. This project was supported by a grant from CAPES, Brazil, and USAID.

**IDENTIFICATION OF A T CELL EPIOTOPE WITHIN THE CS PROTEIN OF
PLASMODIUM VIVAX.**

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Although regions corresponding to T cell epitopes have been described for the CS protein of P. falciparum, similar sequences have not yet been identified for P. vivax. We have obtained circumsporozoite (CS) protein-specific T cell proliferative responses in C3Hf mice (H-2^k) immunized with a purified, recombinant DNA derived P. vivax CS protein (VIVAX 2, Chiron Corp.). In an effort to identify one or more of the T cell epitopes involved in this response, we have synthesized a number of overlapping 15 - 20 amino acid peptides in the vicinity of Region II on the P. vivax CS protein. Our recent immunization studies have focused on four of these peptides designated PV-21, PV-22, PV-23, PV-24. The P. vivax peptides were tested for their ability to evoke a proliferative T cell response in mice. C3Hf mice were inoculated with 100 µg of either peptide or recombinant P. vivax CS protein emulsified in complete Freund's adjuvant. At 2-4 weeks after inoculation, mononuclear leukocytes from the spleens and/or lymph nodes of immunized mice were tested for proliferative responses to CS protein and the various peptides in a six day ³H-thymidine incorporation assay. In mice immunized with CS protein a proliferative response was observed to both CS protein (stimulation index, (S.I.) = 26.9) and PV-23 (S.I. = 4.4). Similarly, mice inoculated with PV-23 were found to respond to both PV-23 (S.I. = 9.0) and the CS protein (S.I. = 3.5). Some mice inoculated with PV-23 also gave a proliferative responses to PV-24 (S.I. = 4.0). Mice inoculated with PV-24 failed to respond to PV-24, but some of these mice did respond to PV-23 (S.I. = 4.0). Since peptides PV-23 and PV-24 share a 10 amino acid region (A.A. 328 - 338), these data indicate that this sequence contains a T cell epitope.

DIFFERENCES IN SUSCEPTIBILITY AMONG MOUSE STRAINS TO INFECTION WITH PLASMODIUM BERGHEI SPOROZOITES AND ITS RELATIONSHIP TO PROTECTION BY GAMMA-IRRADIATED SPOROZOITES.

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Three inbred mouse strains, C57BL/6 (H^2-b), A/J (H^2-a), and BALB/C (H^2-d), and one outbred strain, CD-1, demonstrated differences in susceptibility to challenge with the ANKA clone of Plasmodium berghei. Mice were challenged with 100, 1000, or 10,000 viable sporozoites, then evaluated daily beginning on day four for patency. CD-1 mice were further evaluated at challenge doses of 12,500, 25,000, and 50,000 viable sporozoites. C57BL/6 mice were the easiest to infect with 90% becoming infected with 100 sporozoites. The outbred strain CD-1 was the most difficult to infect requiring 25,000 sporozoites per mouse for a 100% infection rate. Mouse strains also demonstrated differences in their ability to be protected with gamma-irradiated sporozoites. C57BL/6 mice need a minimum of two doses of 10,000 irradiated sporozoites for protection against a challenge with 1000 viable sporozoites. In contrast BALB/C mice, immunized with a single dose of 1000 irradiated sporozoites, are protected and protection are genetically restricted and that susceptibility to infection may be inversely related to protection.

HEAT SHOCK PROTEINS OF PLASMODIUM BERGHEI SPOROZOITES AND EXOERYTHROCYTIC PARASITES.

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Heat shock proteins (hsp) and the 78-kDa glucose-regulated proteins grp78 have been described in eukaryote cells and are encoded by multigene families. Hsp are localized in the nucleus, and may be either expressed constitutively, or are induced by environmental stress, including hyperthermia; grp78 have signal sequences and remain in the lumen of the endoplasmic reticulum (ER). Two P. falciparum genomic clones, one identical (with a few substitutions) to published Pfhsp70, and T-II4 that encodes a protein with a high degree of homology to grp78, have been described (Kumar et al. 1988). T-II4 contains internal homology to grp78 and hsp70 and carboxy-terminal homology to grp78. Interestingly, T-II4 contains an amino acid sequence that resembles the putative hepatocyte binding region of CS proteins. Sera to Pfhsp70, T-II4 and the 11 carboxy-amino acids of T-II4 were tested by immuno-gold electron microscopy with P. berghei sporozoites and exoerythrocytic (EE) parasites and western blots with sporozoites. By electron microscopy and immunoblots, Pfhsp70 was not found in sporozoites, but was found in newly invaded sporozoites, and the nuclei and cytoplasm of P. berghei EE parasites; grp78 was found in the ER of sporozoites and EE parasites. These results suggest that heat shock proteins of P. falciparum and P. berghei are conserved, and that these genes may play a role in the development of these parasites in the invertebrate vector and the vertebrate host.

This project was supported by contracts from USAID.

PREVALENCE, INTENSITY AND NATURAL BOOSTING OF SERUM REACTIVITY TO CIRCUMSPOROZOITE CONSTRUCTS OF PLASMODIUM VIVAX, PLASMODIUM FALCIPARUM, AND PLASMODIUM BRASILIANUM.

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Serum reactivity of 275 inhabitants of a stable P. vivax, P. falciparum, P. malariae hyperendemic area of Malaysia was measured against three circumsporozoite (CS) constructs [NSI₈₁V2O, R₃₂tet₃₂, (DAGN)₃ (AAGN)₃] on the day of radical treatment and each week thereafter for 15 weeks. At treatment the point prevalence of sporozoite induced reactivity to each CS construct was 70% or greater in subjects five years of age or older. Thereafter, natural boosting of the intensity of serum reactivity occurred in both children and adults and reflected the rates and species of sporozoites identified among vector mosquitoes in the study area. The half-life of booster induced CS specific serum reactivity was approximately 28 days for each construct. A consistently positive correlation between reactivity, intensity, natural boosting, and the induction of either a pre-erythrocytic or erythrocytic stage protective immune status was not evident.

VARIATION IN THE PLASMODIUM FALCIPARUM CIRCUMSPOROZOITE PROTEIN GENE: IMPLICATIONS FOR VACCINE DEVELOPMENT.

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The circumsporozoite protein (CSP) is the major candidate for a sporozoite malaria vaccine. While the overall structure and organization of the P. falciparum CSP is similar in different lines, it is becoming clear that the potential for variation is greater than was first apparent. The DNA sequence of the CSP genes from two cloned Thai lines, T9-98 and T9-101 has been determined. The T9-98 gene contains a 57bp deletion in the 5' coding region at a site where a 30bp insertion is found in some strains. The central immunodominant repeat region of the T9-101 CSP gene shows a novel interspersed pattern of major and minor repeats. Amino acid substitutions have been mapped to two areas of the 3' coding region shown to be human immunodominant T-cell sites. The pattern of substitutions differs between the two lines, and no two CSP sequences to date contain the same substitution at each position. The coincidence of these substitutions with putative functional regions and immunodominant T-cell epitopes is disturbing in its implications for development of an effective sporozoite vaccine.

CELL ADHESION TO SYNTHETIC PEPTIDES FROM REGION II OF *P. vivax* CS PROTEIN.

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Region II of the circumsporozoite (CS) protein of *P. vivax* contains sequences related to thrombospondin, a molecule known to exhibit cellular adhesive properties. We tested a series of overlapping, synthetic 15–20AA peptides (designated PV-21, PV-22, PV-23 & PV-24) corresponding to sequences in the vicinity of Region II on the CS protein of *P. vivax*, for their ability to promote attachment of cells to peptide-coated substrates. Cells were labelled with ^{51}Cr and then added to EIA microtiter plates coated with peptides or BSA (control). In some cases, ^{51}Cr -labeled cells were pre-incubated with peptides (3–100 g/ml) for one hour at 37° prior to addition to the microtiter plates. After a 1.5 hour incubation at 37° the non-adhered cells were removed, and the individual wells were counted. Peptides PV-22 and PV-23, but not PV-21 or PV-24, promoted attachment of a variety of human tumor cell lines which grow in suspension culture. T cell-derived lines (CEM & HSB-2) and the myeloid line K562, showed the highest levels of binding (46–76%), while B cell lines (IM-9 & RPMI-7666), the monocytoid line U937 and other epithelial tumor cell lines (H82 and H446, both SCLC) bound only 8–24%. Binding of all cell lines to PV-21, PV-24 or BSA was $4 \pm 2\%$. Furthermore, binding of CEM or K562 cells to PV-22 or PV-23 coated substrate was blocked by preincubation of the cells with either PV-22 or PV-23 (70–95% inhibition). Pre-incubation of cells with PV-21 or PV-24 had minimal effect on subsequent binding to PV-22 or PV-23 coated substrate (10–20% inhibition). While cell adhesion to substrate coated with an equal amount (by weight) of purified recombinant CS protein (Vivax 2, Chiron Corp.) was low (20%), preincubation of cells with PV-22 or PV-23 abolished attachment. Since PV-22 and PV-23 share a region of 10 AA, we proposed that this overlapping sequence mediated cell adhesion. To test this hypothesis, a 10 AA peptide (PV-22/23) corresponding to this overlap region was synthesized. A control peptide of 8 AA (PV-23/24), corresponding to the overlap of PV-23 and PV24, was also synthesized. Preincubation of CEM or K562 cells with PV-22/23 resulted in 72% inhibition of binding to PV-23 coated substrate, while preincubation with PV-22/23 resulted in 72% inhibition of binding to PV-23 coated substrate, while preincubation with PV-23/24 was without effect.

In conclusion: We have demonstrated that peptides corresponding to Region II of **P. vivax** CS protein have cell adhesive properties and thus possess intrinsic biologic activity. Their role in the cellular biology and immunology of parasite infection needs to be investigated.

IMMUNE RECOGNITION OF DOMINANT T CELL-STIMULATING EPITOPES FROM THE CIRCUMSPOROZOITE PROTEIN OF PLASMODIUM FALCIPARUM AND RELATIONSHIP TO MALARIA MORBIDITY IN GAMBIAN CHILDREN.

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A study conducted among malaria-immune adults in a rural area of The Gambia, West Africa in 1987 (Good et al 1988, PNAS 85 1199 - 1203) identified two dominant T cell-stimulating epitopes (Th2R and Th3R) in the circumsporozoite protein (CSP). Both epitopes lie within a region of the molecule (residues 326-380) which is known to vary between parasite isolates. Antibody-independent immune mechanisms seem to be involved in antisporezoite immunity but little is known about the importance of naturally acquired cellular immune responses to CSP in the development of protective immunity to malaria.

In May 1988, venous blood samples were collected from 380 Gambian children (aged 3 to 8 years). *In vitro* lymphoproliferative responses were measured to three variant forms of both Th2R and Th3R and culture supernatants were assayed for Interferon-gamma by ELISA. Anti-CSP antibodies (anti-NANP₄₀). Were measured by ELISA. All the children were monitored during the following malaria-transmission season (June to November 1988) to detect episodes of clinical malaria and subclinical parasitemia. Immune responses were compared with subsequent malaria morbidity in an attempt to evaluate the contribution of anti-CSP immunity to overall immune protection. Preliminary data will be presented.

PLASMODIUM BERGHEI EXOERYTHROCYTIC MEROZOITE SURFACE ANTIGEN: SEROLOGY AND CLONING.

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Considerable serological cross-reactivity occurs between blood stage antigens and those of the exoerythrocytic (EE) stage. Previously, we have shown that anti-P. berghei blood stage sera or MAb's to P. vivax blood stage antigens react in IFA or IPA with P. berghei or P. vivax EE parasites respectively, and that anti-P. berghei blood stage immune sera immuno-precipitate or react in western blots with many P. berghei EE antigens. The major P. falciparum merozoite surface antigen (Pf195) is a candidate malarial vaccine undergoing intensive investigation, and we decided to characterize the homologous antigen of P. berghei. Mice were immunized with P. berghei blood stages, and MAb C2G1 cloned that reacted with the blood stage merozoite surface by IFA. C2G1 reacted with late EE schizonts and not with trophozoites or early schizonts. C2G1 immunoprecipitated a 250 Kd antigen from ^{35}S -methionine metabolically labelled P. berghei blood stages, and a similar 250 Kd antigen from ^{35}S -cysteine labelled P. berghei EE parasites. A P. berghei blood stage mung bean genomic library was screened with C2G1, and a positive clone found. To obtain the insert size, an EcoRI restriction enzyme digestion of C2G1 DNA was performed which showed two bands on agarose gels of 5 and 1.8 Kb, suggesting an insert size of 6.8 Kb. These DNA fragments have been subcloned into puc13 plasmids for sequence analysis. This project was supported by USAID contract DPE-0453-C-00-3051-00.

THE FATE OF THE CIRCUMSPOROZOITE ANTIGENS AND THE EXPRESSION OF THE PRECURSOR OF THE MAJOR MEROZOITE ANTIGENS DURING THE EXOERYTHROCYTIC DEVELOPMENT OF P. BERGHEI.

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The circumsporozoite (CS) proteins and the precursor of the major merozoite antigens (PMMSA) are major candidates for anti-malarial vaccines. Both these antigens are present in the exoerythrocytic (EE) stage. Using monoclonal antibodies against the P. berghei CS (a kind gift from Dr. R. Nussenzweig) and PMMSA proteins and indirect immunofluorescent staining the fate of the CS proteins and the expression of PMMSA was studied during the entire EE cycle of P. berghei, which was cultured in HepG2 cells.

In the early EE parasite the CS proteins were found on the parasite plasmalemma. Prior to segmentation they also localized to vesicles inside the parasite, and during segmentation, to the greatly enlarged parasitophorous vacuole. Immunogold and immunofluorescent staining shows that at the end of the EE cycle the majority of the remaining CS proteins were associated with the spongy stroma in which the emerging EE merozoites lay.

PMMSA first appeared 36–40 hours after sporozoite invasion (the complete cycle takes 48–60 h) when it seemed to localize to the rough endoplasmic reticulum. As the parasite segmented PMMSA developed a circum-cytomeric distribution prior to surrounding individual merozoites. Immunoblotting results showed that the antigen had a similar molecular weight in both stages and was also similarly processed.

The phagocytosis of EE emerging merozoites by Kupffer cells and infiltrating neutrophils and macrophages are likely to be enhanced by antibody against CS and PMMSA binding to the mature liver stage.

FUNCTIONAL ROLES OF T CELL EPITOPES IN THE CS ANTIGEN OF PLASMODIUM FALCIPARUM.

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We have reported previously that cell mediated immune responses to CS antigens may be measured in vivo through the induction of delayed type hypersensitivity (DTH). The T epitope in the repeating domain of the CS protein of P. falciparum induces strong DTH. We assessed the ability of other T epitopes from the CS protein to function in the induction of DTH. Appropriate strains of mice were immunized with the peptide Th2R (containing an H-2^k restricted helper epitope and an H-2^d restricted non-helper epitope), and peptide 368-390 (containing an H-2^k restricted cytotoxic T cell epitope). Upon challenge with the full length CS construct, RI6CSP, none of the mice exhibited significant DTH. To confirm that T cell recognition occurred with the peptides we employed, sera of immunized mice were assayed for antibody activity to R32LR. As expected, CBA mice (H-2^k) immunized with Th2R were primed for a secondary antibody response to the repeat region upon challenge with RI6CSP. Surprisingly, CBA mice immunized with the CTL epitope developed a secondary antibody response greater than that induced by the helper epitope in Th2R. Furthermore, BALB/c mice in control groups immunized with saline exhibited a secondary type antibody response upon challenge with RI6CSP suggesting that H-2^d mice recognize a strong T helper epitope in the CS protein. Interestingly, the IgM antibody responses were markedly reduced in BALB/c mice primed with Th2R and challenged with RI6CSP, possibly as a result of suppression by the non-helper epitope in Th2R. Our results suggest that the T epitopes in Th2R and the CTL epitope of the CS protein of P. falciparum may not function in the induction of DTH. Furthermore, we have observed helper and possibly suppressor functions which have not been described for these epitopes. (Supported by UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases; Gustav and Louise Pfeiffer Research Foundation; Lutheran Brotherhood MD/PhD Scholarship).

STAGE-SPECIFIC RIBOSOMAL RNA PROBES QUANTITATE THE INHIBITION OF SPOROZOITE INVASION (ISI) ASSAY.

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The ISI assay has been suggested as an in vitro quantitative assay of protective anti-sporozoite antibodies (Hollingdale et al 1984) as sera from irradiated sporozoite immunized human volunteers protected to sporozoite challenge, and MAb's to CS proteins that passively protect animals to sporozoite challenge, blocked Plasmodium sporozoite invasion of human hepatoma (HepG2-A16) cells. Currently, inhibition is calculated as the per cent reduction in invaded sporozoites determined microscopically. Recently (Gunderson et al 1987), the stage-specific expression of two genes coding for the small subunit of ribosomal RNA (rRNA) have been shown. The A-genespecific rRNA is predominantly present in blood stages, whereas the C-specific rRNA occurs in sporozoites. Stage-specific A and C rRNA probes have been used to quantitate P. berghei sporozoite invasion and exoerythrocytic development. The A-rRNA probe could selectively detect invaded sporozoites, whereas those attached to the surface of hepatoma cells only reacted with the C-rRNA probe. As few as 10-50 invaded sporozoites could be detected. The ISI activity of MAb to P. berghei CS was similar using rRNA probes and microscopy. The degree of hybridization of the rRNA probe increased exponentially during exoerythrocytic development, whereas hybridization with cultures infected with irradiated sporozoites barely increased, suggesting that the machinery of gene replication may be damaged by irradiation. Use of rRNA probes is faster and potentially more accurate than microscopy allowing multiple sera to be tested in one hybridization experiment. This project was supported by USAID contract DPE-0453-C-00-3051-00.

PLASMODIUM FALCIPARUM NON-CS PROTEIN PRESENT IN SPOROZOITES AND EXOERYTHROCYTIC PARASITES.

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A *P. falciparum* liver stage specific antigen (LSA) was recently cloned and a 196 bp insert sequenced (Guerin-Marchand et al 1987), revealing at least three repeats of the 17 amino acid sequence EQQSDLEQERLAKLEKLQ. We have used this sequence to show that LSA is also a sporozoite antigen that elicits protective anti-sporozoite antibodies by ISI. Two peptides were synthesized: EQQSDLEQERLAKEKLQ and LEQERLAKEKLQEQQSD and coupled through T to tetanus toxoid (TT) at either terminus. Mice were immunized using FCA/FIA, and sera tested by ELISA using uncoupled immunogen, IFA and ISI. EQQ.. vaccines elicited antibodies that only recognized EQQ.. peptides, and LEQ.. vaccine antibodies only recognized LEQ.. peptides. TT coupled to LEQ.. through the N'-terminus was the most immunogenic by ELISA, whereas TT coupled to the carboxy terminus of LEQ.. elicited ELISA antibodies in only 2/20 mice. Epitope mapping revealed two epitopes, and ISI activity to *P. falciparum* sporozoites was correlated with one epitope. Sera also reacted with *P. falciparum* sporozoites by IFA, and with the sporozoite surface by immuno-gold electron microscopy, suggesting that LSA is present in sporozoites and EE stages. Cross-reactivity with *P. berghei* sporozoites was observed but immunoblots suggested that this was from reactivity with *P. berghei* CS proteins. This project was supported by contracts from USAID.

**A MALARIA HEAT SHOCK LIKE PROTEIN EPITOPE EXPRESSED ON THE
INFECTED HEPATOCYTE SURFACE IS THE TARGET OF ADCC MECHANISMS.**

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Cultured hepatic stages of *P. falciparum* and *P. yoelii* allowed us to determine that the 1C11 Mab, which recognizes a C-terminal fragment of the heat shock like, protein HSP70-1, detected an epitope localized on the infected hepatocyte surface. This capability, together with the fact that HsP70 proteins have been shown to play a role in the adaptation of *Leishmania* and *Trypanosoma* in their life cycles, led us to investigate the possible functional and/or immunological roles of this epitope. Among the potential roles, its participation in ADCC mechanisms was investigated. Spleen cells from normal C3H/He mice (Killer: target ratio 10 and 30) were deposited concomitantly with various dilutions of the 1C11 Mab on *P. yoelii* hepatic cultures of 24 hrs, the period necessary for the appearance of the antigen on the cell surface. The examination of cultures stopped at 48 hrs showed that 25 % of the schizonts were specifically and regularly lysed when the killer: target ratio 30 was used.

**HUMAN HEPATIC MEMBRANE PROTEINS 20 kD AND 55 kD DO SPECIFICALLY
BIND PLASMODIUM FALCIPARUM SPOROZOITES.**

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To determine proteins on human hepatocytes which may be involved in the invasion of P. falciparum sporozoites, plasma membranes were isolated from human livers, the proteins were extracted with CHAPS and labelled with ^{125}I /IODOGEN. After incubation of these radiolabelled proteins with P. falciparum sporozoites we detected two bands, 20 kD and 55 kD, by SDS/PAGE-autoradiography. We electrophoretically purified protein fractions of 20 kD and 55 kD and labelled them with ^{125}I /IODOGEN. In binding experiments we show that the binding of both proteins of P. falciparum sporozoites can be strongly reduced by an excess of the corresponding non-labelled proteins. We studied several human and rat membrane proteins of P. falciparum sporozoites. Sheep antisera raised against the 55 kD fraction (B29) react on Western blot only with the human 55 kD protein and give no cross-reaction with rat liver membrane proteins or human kidney membrane proteins. The antiserum B 29 does not cross-react with a human liver 20 kD protein. In some preliminary experiments the protein fractions 20 kD and 55 kD give reduction of invasion of sporozoites into HepG2A16-cells. These results support the assumption that the 20 kD and the 55 kD protein represent the putative hepatic receptor for P. falciparum sporozoite invasion into human hepatocytes. Supported by AID contract DPE-0453-C-00-3051-00.

ULTRASTRUCTURE OF MATURE EXOERYTHROCYTIC FORMS OF PLASMODIUM FALCIPARUM IN THE CHIMPANZEE.

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Mature exoerythrocytic forms (EEP) of P. falciparum from the chimpanzee were examined by light and transmission electron microscopy in a liver biopsy at day six after sporozoite inoculation. Infectivity of the sporozoites obtained from mosquitoes membrane-fed on cultured gametocytes was about 4-6%. In comparison, sporozoites added to human hepatocytes in vitro had only a development percentage of 0.002 to 0.007. The EEF found in the liver biopsy were not all at the same level of development. Immature compact parasites were seen next to stages with fully formed merozoites, indicating a rapid final maturation. The process of merogony appeared to be comparable to that described for other plasmodial species. At day 7 1/2 days already large amounts of rings were seen in the peripheral blood indicating a duration of the liver development of P. falciparum in the chimpanzee of about 6-6 1/2 days. The process of merogony at the fine structural level was roughly comparable to that described for rodent- and other primate parasites in vivo. Compared to the fine structure of liver stages obtained in vitro in cultured human hepatocytes, the parasites described here are much more mature at the same day after infection. There appears to be some cell infiltration with collagen deposition around the intracellular parasite; however, no marked degeneration was observed.

BOOSTING CAPACITY OF R₁₆HBsAg-VACCINE IN MICE PREVIOUSLY INFECTED WITH P. FALCIPARUM SPOROZOITES.

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Aim of the present study was to investigate whether the candidate sporozoite vaccine R₁₆HBsAg (SKF) was able to boost an anti-NANP antibody response in mice that had been exposed to sporozoites of P. falciparum. This might give an indication whether this putative vaccine may contribute to malaria control in areas with seasonal transmission. Production of antibodies to the repeat of the circumsporozoite (CS) protein (NANP) are controlled by genetically restricted T-cell responses. C57BL/6 mice (H-2^b) recognize T-cell sites on NANP, while BIO.BR (H-2^k) show no T-cell reactivity to the repeat and subsequently do not mount an antibody response unless a carrier is coupled to this repeat (1). Both groups of mice were i.v. injected with live sporozoites. Upon a challenge with R₁₆HBsAg only the H-2^b mice showed a booster effect on anti-NANP antibodies. Non-responsiveness of the BIO.BR mice could be overcome by immunization with HBsAg vaccine after the sporozoite injection. Thus, separate injection of CS-hapten and HBs-carrier induces responsiveness to NANP. The implications will be discussed.

HUMORAL IMMUNE RESPONSE TO THE CIRCUMSPOROZOITE PROTEIN OF PLASMODIUM VIVAX.

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The presence of a humoral immune response to central repeat region of the circumsporozoite protein of P. vivax was determined in residents of a malarious area in the jungle of northern Peru. Only P. vivax malaria is found in this area. An ELISA was used to measure serum IgG antibodies against the recombinant circumsporozoite protein, NSIV20. The prevalence of IgG antibodies to NSIV20 increased with age, being the highest in those individuals 15 years of age and older. The prevalence of blood stage infections with P. vivax was greatest in the 2-9 year olds.

Age Group	Percent	Ab Positive	Percent	Slide Positive
<2	0	(0/16)	4.7	(1/27)
2 - 9	3	(3/91)	18	(19/105)
10 - 15	9	(4/44)	11	(7/63)
<15	23	(30/121)	7.6	(10/132)

This population is currently being followed in a prospective study of the cellular and humoral immune response to recombinant and synthetic circumsporozoite proteins of P. vivax.

PLASMODIUM BERGHEI: EXPRESSION OF CIRCUMSPOROZOITE PROTEIN DURING EXOERYTHROCYTIC DEVELOPMENT IN HEPG2-A16 HEPATOMA CELLS.

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The fate of P. berghei circumsporozoite (CS) protein was studied by post-embedding immunoelectron microscopy in exoerythrocytic (EE) stages grown in HepG2-A16 hepatoma cells. We used monoclonal antibody (MAb) 3D11 against the P. berghei CS protein to localize CS antigen in developing parasites at 3, 24, 50 and 70 hours after sporozoite invasion. CS antigen or a cross reacting epitope persisted throughout EE development as a major schizont surface protein and was incorporated into the membrane of budding EE merozoites. Erythrocytic merozoites were not labeled by MAb 3D11, indicating a clear antigenic difference from EE merozoites. Significant internal labeling within 50 hour EE schizonts was associated with membrane-bounded vesicles of various sizes and with electron-dense patches under the plasma membrane. MAb 3D11 labeled the outer membrane of the internal vesicles but not an electron-dense flocculent material within their contents. Similar unlabeled flocculent material was also present in the parasitophorous vacuole (PV) space and in vesicles in the host cell cytoplasm which were adjacent to the PV. These observations suggest that P. berghei CS protein may play an important role in mediating host-parasite interactions during EE development.

FAILURE OF VACCINIA, PSEUDORABIES AND SALMONELLA CS CONSTRUCTS TO INDUCE PROTECTIVE CELL MEDIATED IMMUNITY AGAINST SPOROZOITES OF PLASMODIUM YOELII.

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In an attempt to induce a protective cell mediated immune response against sporozoites of P. yoelii, the gene encoding the P. yoelii CS protein was engineered into three live vectors, vaccinia, pseudorabies, and attenuated Salmonella typhimurium. BALB/c mice were immunized with four doses of 10^9 pfu of the vaccinia construct (IP), three doses of 10^7 pfu (IV) of pseudorabies construct, and three doses of 10^9 (per os) of the Salmonella construct. In the case of vaccinia and pseudorabies constructs, a strong immune response was obtained as measured by antibodies to sporozoites. No protection or delay in prepatent period was seen in any of the experimental animals challenged with 200 (in the case of Salmonella 100) sporozoites, although mice immunized with irradiation attenuated sporozoites are consistently protected against challenge with $>10^4$ sporozoites. It is currently unclear if the vaccines did not induce the appropriate cellular immune response against the CS protein, or if such an immune response against the P. yoelii CS protein is inadequate to protect against sporozoite challenge.

HEPATIC STAGES OF MALARIA IN CULTURE: A HELPFUL BUT LIMITED TOOL TO DECIPHER PROTECTION MECHANISMS.

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After an infatuation with antisporezoite antibodies, which has led to the development of numerous inhibition assays, the current trend is not to use hepatic cultures to try to decipher the cellular response. With more sophisticated systems, including co-culture of hepatocytes and non parenchymal cells, attempts have been made to reproduce the in vivo situation more closely. These in vitro models provide the means of 1) analyzing the effects of cytokines, TNF, IFNs, IL-1, IL-6..., such as their direct or indirect modes of action resulting from a cascade of events, 2) determining the cytotoxic capability of stimulated T-cells isolated from mice immunized with recombinant or synthetic putative T-cell epitopes, 3) evaluating the role of epitopes expressed at the surface of infected hepatocytes, for example, in relation with ADCC mechanisms. It is nevertheless clear that culture systems, even sophisticated, cannot faithfully mirror the in vivo situation. In no way can in vitro results be used to predict the level of protection in vivo. This implies that, parallel with in vitro experiments, useful for pinpointing the mechanisms involved, in vivo studies must be performed.

GENETIC RESTRICTION OF IMMUNITY TO PLASMODIUM YOELII SPOROZOITES.

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We immunized a series of congenic mice with irradiated P. yoelii sporozoites and found only 2 of 10 strains had strong protective responses. Analysis of BALB and C57BL/10 congenics indicated that genes inside and outside the H-2 region controlled this immunity. BALB/c mice were protected by CD8⁺ effector T cells, but congenic mice on the C57BL/10 background were protected by a mechanism independent of CD8⁺ cells.

This genetic restriction of responses to P. yoelii sporozoites contrasts with the ability of irradiated P. berghei sporozoites to strongly protect all strains of mice tested. We discuss possible reasons for this difference, and relate this to data on primate and human malaria.

DESIGNING PROTEOSOME-PEPTIDE VACCINES TO INDUCE ANTIBODIES AGAINST THE HIGHLY CONSERVED PENTAPEPTIDE (N1A) IN REGION ONE OF MALARIA CIRCUMSPOROZOITE (CS) PROTEINS: THE EFFECTS OF REPLICATING THE HYDROPHOBIC ANCHOR AND/OR N1A.

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Region I of the CS protein of many strains of malaria contain a highly conserved pentapeptide, KLKQP. This sequence, N1A, is at the amino terminus of the N1 family of peptides which have been identified as the putative sporozoite receptor for hepatocyte recognition and invasion since it shows saturable binding to hepatocytes by Scatchard analysis. Preliminary data have indicated that active binding site is contained within this ultraconserved sequence, KLKQP. Thus, antibodies to N1A should protect against malaria by preventing sporozoite invasion. It was therefore considered important to develop vaccines to induce antibodies that exclusively recognize this peptide. Initial experiments using conventional covalent methodology to link the peptide to carrier proteins were largely unsuccessful in inducing N1A antibodies. We have previously shown that adding a hydrophobic anchor and cysteine to peptides and hydrophobically complexing them to meningococcal outer membrane protein proteosomes highly enhances their immunogenicity. Accordingly, a series of peptides containing N1A were synthesized (by t-boc or f-Mocchemistry) with cysteine and hydrophobic amino acid anchors. Peptide immunogenicity is greatly influenced by the length and orientation of both the peptide and the linking hydrophobic anchor. To determine the construct that most effectively induces N1A antibodies, proteosome vaccines were designed to contain from one to four tandemly repeated copies of the N1A pentapeptide [(N1A)_n] and one to three tandemly repeated copies of the hydrophobic heptapeptide anchor, FLLALLF [(Ft)_n] added to either the amino or carboxy terminal of (N1A)_n. After two immunizations of CD-1 (outbred) and BALB/c mice with 12 of these constructs complexed to proteosomes, it was found that the most immunogenic vaccines contained (N1A)₄-(Ft)₂ or (N1A)₄(Ft)₁. These vaccines induced specific antibodies when given in either normal saline or CFA. In contrast, other constructs lacked significant activity. These data show that the proteosome-hydrophobic anchor system can be used to make a pentapeptide immunogenic and that the epitope: anchor ratio in such vaccines is critically important. These vaccines are directly applicable for immunogenicity and

protection studies in mice, monkeys and humans using P. berghei, P. cynomolgi and P. falciparum, respectively, since these strains are homologous for NIA.

A RECOMBINANT *P. FALCIPARUM* CIRCUMSPOROZOITE (CS) PROTEIN (R32Ft) DESIGNED WITH A HYDROPHOBIC DECAPEPTIDE ANCHOR: PURIFICATION AND IMMUNOGENICITY IN CS - REPEAT RESPONDER AND NON-RESPONDER MICE EITHER WITHOUT ADJUVANTS OR HYDROPHOBICALLY COMPLEXED TO PROTEOSOMES.

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We have previously shown that by adding a carboxylic or hydrophobic amino acid anchor containing cysteine to a small peptide, it can become immunogenic in mice and that the immunogenicity of such anchored peptides is highly enhanced by hydrophobically complexing them to meningococcal outer membrane protein proteosomes. R32LR, a recombinant product with 32 tandem tetrapeptide repeats derived from the *P. falciparum* CS protein is not immunogenic unless given with an adjuvant (e.g. CFA) to responder (H-2b) mice (e.g. C57Bl/6). Covalent coupling of R32LR to another protein has previously been shown to enhance its immunogenicity. In order to add a hydrophobic anchor to R32LR, a synthetic nucleotide encoding for a series of 10 hydrophobic amino acids was added to the 3' end of the gene. This protein, R32Ft, was very well expressed in *E. coli* and was purified using a rapid, sensitive ELISA and modifications (to account for its hydrophobic anchor) of the scheme described for R32LR. Amino acid analysis confirmed the purity of R32Ft after preparative C3 RP-HPLC gel filtration.

In contrast to R32LR, R32Ft was immunogenic without adjuvants even in H-2d mice (BALB/c) which are genetically restricted from responding to the CS-repeat epitope. Moreover, complexing R32Ft to proteosomes markedly enhanced its immunogenicity without any other adjuvants in all mouse strains tested. Small lipopeptides (containing four or six CS tetrapeptide repeats) hydrophobically complexed to proteosomes via lauryl-cysteine hydrophobic anchors were also immunogenic in CS-repeat non-responder strains. Unlike R32Ft, however, small lipopeptides in saline without proteosomes were not immunogenic in non-responder strains. These data show that a hypo-immunogenic, genetically-restricted protein can become immunogenic without other adjuvants even in a previous non-responder mouse strain by genetically engineering it to contain a decapeptide carboxy-terminal hydrophobic anchor. Furthermore, these data demonstrate that proteosomes can also be used to enhance the immunogenicity of a recombinant protein when hydrophobically

complexed to it via its hydrophobic anchor. Since R32Ft is readily purified, well-expressed and highly immunogenic when complexed to proteosomes, it is a prime candidate as a human vaccine component to enhance immunity against P. falciparum sporozoites.

CLINICAL TRIALS WITH SPOROZOITE VACCINES—WRAIR

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Protection of mice by passive transfer of monoclonal antibodies directed against a repetitive epitope on the circumsporozoite (CS) proteins of P. berghei and P. yoelli has convincingly demonstrated the ability of antibody to prevent sporozoite-induced malaria. One major focus of our sporozoite vaccine program is the development of vaccines designed to elicit high levels of protective antibodies directed against the repetitive CS epitopes of P. falciparum and P. vivax. Immunization of humans with our first subunit vaccine produced modest levels of antibody that conferred partial protection as manifested by a prolonged pre-patent period in two of six and complete protection in one of six after laboratory challenge. In an attempt to elicit higher levels of antibodies in a larger percentage of vaccines we have tested several vaccines, each in a small number of volunteers. These vaccines include recombinant expression proteins either alone or conjugated to carrier proteins as well as synthetic peptides coupled to carrier proteins. Some of these have increased the average antibody level two - three fold over our initial construct. The presentation will review these results.

IN VITRO EVALUATION OF A TEST WHICH MEASURES THE ACTION OF ANTIBODIES ON THE PRE-ERYTHROCYTIC STAGES OF MALARIA PARASITES.

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We have evaluated the in vitro biological activity of antibodies directed against sporozoites and compared it with their capacity to protect against challenge with both human and rodent malaria. Since sporozoites must enter hepatocytes to transform to next stage of development, we assessed the effect of antibodies on sporozoites invasion and development in hepatocytes in vitro. In the Inhibition of Liver Stage Development Assay (ILSDA), we first showed that two monoclonal antibodies to Plasmodium falciparum sporozoites inhibited parasite invasion and development by concentration dependent fashion and total inhibition only occurred at high concentration (10–100 (μ) g/ml). However, sera from humans living in hyperendemic areas by IFAT (10⁻⁵), could never be shown to inhibit more than 88%. To further assess the correlation between ILSDA results and protective immunity, we moved to the P. yoelli rodent malaria model system. Mice that received a monoclonal antibody to the P. yoelli circumsporozoite protein (CS protein) were 100% protected against challenge with 5000 sporozoites, but mice immunized with subunit P. yoelli CS protein vaccine that had comparable levels of antibodies to sporozoites and CS protein by IFAT and ELISA were not protected against challenge with 40–200 sporozoites. In the ILSDA, sera from protected mice inhibited liver stage development by 100% at a dilution of 1/10 and 91% at 1/100, while the most active sera from unprotective mice inhibited 91% at 1/10 and 0% at 1/100. These data suggest that in contrast to ELISA and IFAT, ILSDA may be an appropriate test to evaluate the biologic relevance of an antibody component.